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Heat hardening capacity in *Drosophila melanogaster* is life stage specific and juveniles show the highest plasticity

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Abstract

Variation in stress resistance and adaptive plastic responses during ontogeny have rarely been addressed, despite the possibility that differences between life stages can affect range margins and thermal tolerance of species. Here we assessed the thermal sensitivity and hardening capacity of *Drosophila melanogaster* across developmental stages from *larval* to the adult stage. We observed strong differences between life stages in heat resistance with adults being most heat resistant followed by *puparia*, *pupae* and *larvae*. The impact of heat hardening (1h at 35 °C) on heat resistance changed during ontogeny with the highest positive effect of hardening observed in *puparia* and *pupae* and the lowest in adults. These results suggest that immobile life stages (*puparia* and *pupae*) have evolved high plasticity in upper thermal limits whereas adults and *larvae* rely more on behavioral responses to heat stress allowing them to escape from extreme high temperatures. While most studies on the plasticity of heat resistance in ectotherms have focused on the adult life stage, our findings emphasize the crucial importance of juvenile life stages of arthropods in understanding the thermal biology and life stage specific physiological responses to variable and stressful high temperatures. Failure to acknowledge this complication might lead to biased estimates of species' ability to cope with environmental changes, such as climate change.

Introduction

Adaptive phenotypic plasticity is a mechanism enabling organisms to adjust their phenotype to changing conditions and this is proposed to be especially important in fluctuating environments [1, but see 2]. The induction of plastic responses can occur through hardening where a brief exposure to a non-lethal condition triggers changes, that can increase the ability of organisms to tolerate subsequent more extreme conditions [3]. For example, heat or cold hardening induces plastic physiological and behavioral responses that significantly affect the ability to tolerate subsequent more extreme high or low temperatures and this seem to be a general phenomenon across a wide range of organisms [4–6].

In holometabolous insects, each life stage may have a different capacity for plasticity due to variation in the thermal sensitivity of life stages and/or morphological and physiological differences between them [7]. For example, low mobility and lack of fully functional organs during pre-adult stages may increase the selection pressure on plastic responses that improve the thermal tolerance in the juveniles. However, adults may show a lower thermal plasticity as a consequence of their high dispersal ability that allow them to avoid extreme conditions [8].

The influence of physiological or morphological changes induced by hardening or acclimation on thermal tolerance is a well-studied phenomenon, particularly in ectotherms [9]. However, most published studies on insects focus on adults, whereas plasticity of other life stages and its importance in mediating responses to daily and seasonal thermal fluctuations has rarely been addressed [10–12]. Such information is however key to understanding the range- and tolerance limits of species, as knowledge from a single life-stage could over- or underestimate species tolerance. Thus, this can hinder our ability to correctly predict the consequences of altered environments, for example due to climate change, on distributions and future prospects of species [7]. Here, we conducted an experiment with *Drosophila melanogaster* in which the heat resistance of hardened and non-hardened individuals was assessed across seven developmental stages (3 *larval*, *puparium*, *pupa*, and 2 adult stages). We hypothesized that sessile life stages (*puparium* and *pupa*) or stages with low mobility (*larva*) show higher plasticity in response to heat hardening compared to adults, which are better able to evade adverse conditions by dispersal.

Materials and Methods

Population

A *D. melanogaster* population was set up in 2010 using the offspring of 589 inseminated females caught at Karensminde fruit farm in Odder, Denmark (55°57´ N, 10°09´ E). The population was maintained on standard *Drosophila* agar-sugar-yeast-oatmeal medium at 25 ± 1˚C and on a 12h light:12h dark cycle [13]. For the sample collection, adult flies (6 to 7 days old) were placed into 300 mL plastic bottles containing a plastic spoon filled with 5 mL standard medium (50 to 60 flies per bottle, 20 bottles per sampling period). Unless otherwise stated, flies were allowed to lay eggs for 2h, thereafter eggs were collected at a controlled density (15 eggs per 35 mL plastic vial containing 7 mL standard medium) and kept at 25 ± 1˚C and on a 12h L:12h D cycle until they reached the specific life stage being investigated (see below).

Larvae (1^{st}, 2^{nd} & 3^{rd} instar *larvae*): *larval* stages were defined by the time after oviposition. The first, second and third instar *larvae* were collected 24, 48 and 72h after oviposition, respectively. The selected stages are physiologically, morphologically, and behaviorally different from each other. The first two *larval* stages mainly search for food and eat while the third instar larvae crawl out of the food source to search for a suitable pupation site. At each stage, 10 *larvae* were collected into each of 180 vials with 7 mL standard *Drosophila* medium.

Puparia and pupae: for both *puparial* and *pupal* stages, 15 eggs were collected into each of 180 35 mL vials containing 7 mL standard *Drosophila* medium. 96h after egg collection the vials were inspected and the few early-formed *puparia* (rarely observed) were gently removed from vials and discarded to control the age of samples. 122h (*puparium*) or 168h (*pupa*) after oviposition, the number of *puparia* or *pupae* in all vials was counted.

Adult (1- & 3-day old): The flies were collected 24h after the first emergence and placed into 35 mL plastic vials containing 7 mL standard *Drosophila* medium. For both ages, we placed 10 flies per vial, pooled sexes. We did not separate male and female adult flies, to match the handling of juvenile life stages where we did not know the distribution of males and females in the test samples.

Thermal sensitivity

Heat tolerance was tested for all life stages using heat mortality assays exposing flies to six different test temperatures (25, 37, 38, 39, 40 and 41 ºC) with or without prior hardening (1 h at 35 ºC). Pilot studies were conducted to determine appropriate hardening and test temperatures as well as their duration (data not shown). The selected heat hardening temperature and duration were sufficient to induce a heat stress response [14] but did not cause mortality in any of the life stages. The test temperatures reduced survival markedly, at least at the highest test temperature, after one hour exposure. All individuals were tested in 35 mL plastic vials containing 7 mL standard *Drosophila* medium providing an environment where the temperature changed gradually to reach the test temperature. At each life stage half of the collected samples (90 vials out of 180) were placed in a water bath set at 35 ºC for 1h (heat hardening) and the rest of the vials were kept at 25 ºC. Thereafter, equal numbers of hardened and non-hardened vials with individuals were randomly assigned to six water baths (15 replicate vials per treatment) set at 25, 37, 38, 39, 40 or 41 ºC. The samples were exposed to the test temperature for 1h and then placed in a climate room (25 ± 1˚C and 12h L:12h D cycle). Adult flies were scored for survival 24h after the heat treatment. For the remaining life stages vials were kept in the climate room (25 ± 1˚C and 12h L:12h D cycle) until adults emerged. Upon emergence flies were counted (not sexed) and removed each day until no new flies had emerged for 3 consecutive days.

Data analysis

For all life stages, the proportion of survivors from each vial was calculated as the number of live flies divided by the sum of dead and alive flies in each vial. The mortality rate at 25 ºC and 37 ºC test temperatures with or without hardening displayed a similar pattern throughout ontogeny (Table S1). Therefore, data on survival at 25 ºC was removed from the dataset, to improve the data fit. The influence of hardening on thermal resistance of individuals throughout ontogeny was investigated using a linear model with hardening and life stage as fixed factors, with test temperature as a continuous variable, and including all interactions between fixed and continuous factors. We also removed the hardening factor from the model and analyzed the heat resistance of only non-hardened flies to test the life stage-specific basal thermal tolerance. In both analyses, the test temperature was mean centered (mean temperature minus each of the test temperatures) and the survival proportion was arcsine-square-root transformed. P-values were adjusted for multiple pairwise comparisons using a false discovery rate at the 5% level [15]. All analyses were performed with R (version 3.4) and RStudio (version 1.1.44).

Results

The impact of hardening on heat resistance varied significantly between life stages and test temperatures (hardening × life stage × test temperature: $F = 23.67$, df = 6, $p < 0.0001$). *Puparium* and *pupa* responded most to hardening illustrated by a relatively constant survival across different test temperatures (~ 97% survival on average) while the non-hardened groups displayed a reduction in survival from 39 °C onwards (Fig. 1, Table 1). The hardened and non-hardened *larvae* (all three stages) showed a similar survival pattern with significantly higher resistance of the hardened group mainly at temperatures above 37 ºC. Hardening did not affect the thermal resistance of 1-day old adults while at 3 days of age, hardening significantly reduced the thermal resistance of flies at 40 and 41 ºC. Within hardened or non-hardened groups, the heat resistance varied between life stages in a temperature-specific manner (non-hardened: $F = 5.64$, df = 6, $p < 0.0001$; hardened: $F = 40.51$, df =

6, $p < 0.0001$, Table S2). In general, the non-hardened adults showed a significantly higher survival than *puparia* and *pupae* especially at 40 and 41 ºC. The hardened *puparia* and *pupae* were more heat resistant than the hardened adults (both ages) across the test temperatures except at 38 ºC, where no difference was observed between adults (both ages) and *puparia* as well as *pupae* (Table S2).

Discussion

As hypothesized, we observed that adaptive hardening responses were most pronounced in more sessile life stages compared to mobile adults. Under the hardening and test conditions we used, *puparia* and *pupae* followed by *larvae* (all three stages) had very strong hardening capacity compared to adults, where hardening either had no (1-day old adults) or negative (3-day old adults) effect on thermal resistance. These findings may arise from the ability of adults to evade critically extreme temperatures through behavioural responses and hence dismissing the need for responding plastically to quickly changing temperatures. Therefore, our data suggest, that in thermal variable environments natural selection will favor individuals / genotypes that are plastic as juveniles and less plastic but good dispersers at adult life stages [16]. The basal heat resistance was higher in adults than in other life stages (Fig. 1), which may be linked to the stage-specific energy allocation strategies in holometabolous insects and difference in energy requirement during ontogeny [17].

The increased survival of the hardened compared to the non-hardened juveniles points to their high dependence on plastic responses in the face of sudden temperature changes. Low plasticity of adults in upper thermal limits is a common observation in the literature [2,18], which can be a strategy to prevent the costs of physiological adjustments in response to thermal variation [4]. The absence of this pattern in juvenile stages, at the conditions that we have tested, highlights the need to perform studies on pre-adult stages to get a more complete picture of the thermal biology of a species. This is currently not a common practice as at least in *Drosophila*, where most studies focus on the adult life stage [but see 19].

Our findings provide evidence that different life stages have different thermal sensitivity and hardening capacity. The results suggest that the ability to cope with adverse thermal conditions has evolved in a life stage-specific manner. Such life-stage specificity in key adaptation mechanisms suggest that concentrating studies on a single life-stage, or single trait, in determining the range limits, or evolutionary potential of a species can bias the predictions concerning the ability to cope with environmental changes, such as climate change.

Ethics

NA

Data accessibility

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Author contributions

NNM, CP, SB and TNK designed and NNM performed the experiment. NNM and TK analyzed the data. NNM, TNK, and TK wrote the manuscript, CP and SB provided useful comments on the manuscript and all authors approved the final version. All authors agree to be held accountable for the content of this manuscript.

Competing interests

We have no competing interests.

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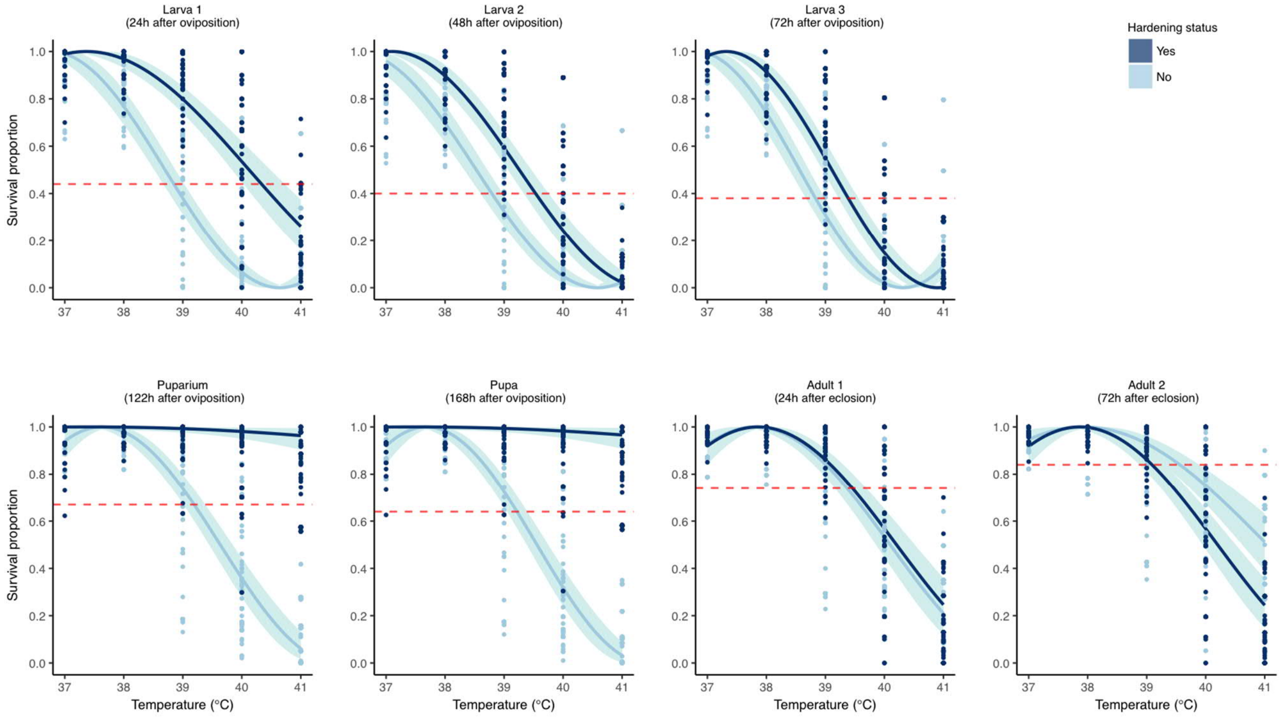
Table and Figure Legends

Table 1. Results from the ANCOVA analysis testing heat resistance of hardened vs. non-hardened groups at different test temperatures throughout ontogeny. The table shows the F_{df} ratio and the p-values with $p < 0.05$ in bold.

Fig.1. Fitted regression lines of the survival proportion of hardened (1h at 35 ºC, dark blue line) vs. non-hardened (light blue line) *D. melanogaster* at different life stages from *larval* to adult after 1h exposure to 37, 38, 39, 40 or 41 °C. The dashed red line shows the basal thermal tolerance (average survival proportion of non-hardened flies across the test temperatures).

Table 1

	Test temperature (°C)									
	37		38		39		40		41	
	F_1	p	F_1	p	F_1	p	F_1	p	F_1	p
Larva 1	5.27	0.06	26.63	**< 0.0001**	75	**< 0.0001**	80.68	**< 0.0001**	59.33	**< 0.0001**
Larva 2	7.29	**0.04**	16.81	**0.0003**	28.80	**< 0.0001**	21.75	**< 0.0001**	12.23	**0.002**
Larva 3	6.62	0.05	14.28	**0.0008**	23.04	**< 0.0001**	16.48	**0.0001**	8.81	**0.006**
Puparium	6.46	0.05	3.30	0.28	78.39	**< 0.0001**	159.80	**< 0.0001**	162.97	**< 0.0001**
Pupa	8.70	**0.02**	2.87	0.28	85.72	**< 0.0001**	180.25	**< 0.0001**	186.09	**< 0.0001**
Adult 1	0.01	1.00	0.09	1.00	0.27	0.60	0.31	0.57	0.24	0.62
Adult 2	0.42	1.00	0.18	1.00	4.75	0.06	9.80	**0.003**	10.03	**0.005**



Larva 1
(24h after oviposition)
Larva 2
(48h after oviposition)
Larva 3
(72h after oviposition)
Hardening status
Yes
No
Puparium
(122h after oviposition)
Pupa
(168h after oviposition)
Adult 1
(24h after eclosion)
Adult 2
(72h after eclosion)
Survival proportion
Temperature (°C)

Fig. 1

Table S1. Tukey's post-hoc test results after false discovery rate correction to compare the heat resistance of hardened and non-hardened individuals at different life stages exposed to 25 ºC compared to corresponding 37 ºC test temperature. The table shows the sum of square (SS), F_{df} ratio and the p-values.

Temperature (ºC)	Life stage	Hardening status	SS	F_1 ratio	p value
25 *vs.* 37	*Larvae 1*	Yes	0.6583	3.20	0.09
25 *vs.* 37	*Larvae 2*	Yes	0.0007	0.36	1
25 *vs.* 37	*Larvae 3*	Yes	0.0035	1.87	1
25 *vs.* 37	*Puparia*	Yes	0.0026	1.43	1
25 *vs.* 37	*Pupae*	Yes	0.0025	1.34	1
25 *vs.* 37	Adult 1	Yes	0.0000	0	1
25 *vs.* 37	Adult 2	Yes	0.0000	0	1
25 *vs.* 37	*Larvae 1*	No	0.0043	2.34	1
25 *vs.* 37	*Larvae 2*	No	0.7208	1.18	0.06
25 *vs.* 37	*Larvae 3*	No	0.0000	0	1
25 *vs.* 37	*Puparia*	No	0.0028	1.53	1
25 *vs.* 37	*Pupae*	No	0.1424	4.21	1
25 *vs.*37	Adult 1	No	0.0000	0	1
25 *vs.* 37	Adult 2	No	0.0035	1.87	1

Table S2. Tukey's post-hoc test results after false discovery rate (FDR) correction to compare the heat resistance of life stage at different test temperatures. The table shows the F_{df} ratio and the p-values with $p < 0.05$ in bold.

		Test temperature (°C)									
		37		38		39		40		41	
		F_1	p	F_1	p	F_1	p	F_1	p	F_1	p
Non-hardened group	*Larva1 vs. Larva2*	1.61	1.00	3.53	1.00	1.26	0.84	2.06	1.00	0.15	0.91
	Larva1 vs. Larva3	0.10	1.00	1.61	1.00	6.52	0.66	1.51	0.30	6.91	0.34
	Larva2 vs. Larva3	3.58	1.00	3.84	1.00	0.65	1.00	3.84	0.64	3.58	0.34
	Larva1 vs. Puparium	5.56	**0.00**	29.45	**0.00**	47.28	**0.00**	33.66	**0.00**	7.23	**0.00**
	Larva1 vs. Pupa	1.11	**0.00**	31.52	**0.00**	43.53	**0.00**	26.63	**0.00**	1.67	**0.00**
	Larva2 vs. Puparium	2.00	**0.00**	44.25	**0.00**	63.37	**0.00**	40.29	**0.00**	2.10	**0.00**
	Larva2 vs. Pupa	27.43	**0.00**	46.78	**0.00**	59.01	**0.00**	32.55	**0.00**	1.93	**0.00**
	Larva3 vs. Puparium	6.30	**0.00**	34.90	**0.00**	68.33	**0.00**	57.62	**0.00**	35.55	**0.00**
	Larva3 vs. Pupa	1.13	**0.00**	37.15	**0.00**	63.81	**0.00**	48.29	**0.00**	27.20	**0.00**
	Larva1 vs. Adult1	2.30	**0.00**	43.91	**0.00**	85.61	**0.00**	71.96	**0.00**	44.28	**0.00**
	Larva1 vs. Adult2	5.27	**0.00**	53.98	**0.00**	139.89	**0.00**	143.21	**0.00**	101.92	**0.00**
	Larva2 vs. Adult1	26.38	**0.00**	61.65	**0.00**	106.85	**0.00**	81.51	**0.00**	46.24	**0.00**
	Larva2 vs. Adult2	6.42	**0.00**	73.49	**0.00**	166.72	**0.00**	156.56	**0.00**	104.87	**0.00**
	Larva3 vs. Adult1	2.49	**0.00**	50.52	**0.00**	113.27	**0.00**	105.51	**0.00**	70.25	**0.00**
	Larva3 vs. Adult2	6.25	**0.01**	61.29	**0.00**	174.71	**0.00**	189.22	**0.00**	139.77	**0.00**
	Puparium vs. Pupa	1.26	1.00	0.24	1.00	0.54	1.00	2.86	1.00	3.88	0.91
	Puparium vs. Adult1	0.73	1.00	3.09	1.00	4.70	0.09	1.59	**0.04**	6.13	0.08
	Puparium vs. Adult2	0.14	1.00	4.91	3.46	24.52	**0.00**	38.01	**0.00**	34.34	**0.00**
	Pupa vs. Adult1	0.07	1.00	0.22	1.00	0.63	0.06	0.74	**0.01**	0.60	**0.01**
	Pupa vs. Adult2	2.24	1.00	3.00	4.65	27.35	**0.00**	46.34	**0.00**	43.65	**0.00**
	Adult1 vs. Adult2	1.52	1.00	3.61	1.00	4.63	0.06	1.47	**0.00**	6.28	**0.00**
Hardened group	*Larva1 vs. Larva2*	4.61	1.00	2.04	0.14	2.65	**0.00**	27.05	**0.00**	4.04	**0.00**
	Larva1 vs. Larva3	1.28	1.00	0.40	0.36	32.07	**0.00**	52.41	**0.00**	48.56	**0.00**
	Larva2 vs. Larva3	3.84	1.00	1.69	1.00	6.22	1.00	1.24	1.46	3.39	0.10
	Larva1 vs. Puparium	6.77	1.00	3.23	0.14	62.74	**0.00**	108.14	**0.00**	102.69	**0.00**
	Larva1 vs. Pupa	6.18	1.00	6.06	0.12	64.62	**0.00**	110.51	**0.00**	104.56	**0.00**
	Larva2 vs. Puparium	0.21	1.00	25.37	**0.00**	159.71	**0.00**	243.37	**0.00**	218.03	**0.00**
	Larva2 vs. Pupa	0.12	1.00	26.18	**0.00**	162.70	**0.00**	246.91	**0.00**	220.75	**0.00**
	Larva3 vs. Puparium	2.01	1.00	5.33	**0.00**	184.52	**0.00**	311.11	**0.00**	292.47	**0.00**
	Larva3 vs. Pupa	6.18	1.00	3.47	**0.00**	187.74	**0.00**	315.12	**0.00**	295.63	**0.00**
	Larva1 vs. Adult1	6.01	0.40	1.08	0.22	3.01	2.81	2.25	1.00	0.27	1.00
	Larva1 vs. Adult2	0.52	0.38	2.29	0.22	0.54	2.81	2.13	1.00	0.35	1.00

Larva2 vs. Adult1	0.99	**0.04**	5.85	**0.00**	41.62	**0.00**	33.29	**0.00**	5.44	**0.00**
Larva2 vs. Adult2	2.92	**0.04**	1.49	**0.00**	41.81	**0.00**	33.12	**0.00**	3.66	**0.00**
Larva3 vs. Adult1	1.07	0.93	2.51	**0.00**	54.73	**0.00**	60.97	**0.00**	45.85	**0.00**
Larva3 vs. Adult2	2.21	0.91	4.79	**0.00**	54.94	**0.00**	60.74	**0.00**	45.46	**0.00**
Puparium vs. Pupa	0.00	1.00	0.01	1.00	0.10	1.00	0.09	1.00	0.01	1.00
Puparium vs. Adult1	1.53	**0.03**	0.51	1.00	38.27	**0.00**	96.64	**0.00**	106.72	**0.00**
Puparium vs. Adult2	3.57	**0.03**	0.38	1.00	38.09	**0.00**	96.92	**0.00**	107.32	**0.00**
Pupa vs. Adult1	6.50	**0.03**	0.85	1.00	39.74	**0.00**	98.87	**0.00**	108.63	**0.00**
Pupae vs. Adult2	1.61	**0.03**	0.68	1.00	39.56	**0.00**	99.16	**0.00**	109.24	**0.00**
Adult1 vs. Adult2	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00